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14. ABSTRACT

We continue to make excellent progress and the data to date are clearly supportive that TLR4 is an important drug abuse target. Toll like receptor 4 (TLR4) continues to present as a powerful modulator of drug abuse as blocking .TLR4 with (+)naltrexone or (+)--.naloxone suppresses multiple indices of drug reward and drug reinforcement for both opioids and cocaine. It is exciting that we are documenting the sites of action of TLR4 on drug abuse to the NAc shell and VTA, key structures in the rewarding and reinforcing effects of cocaine and opioids, the abused drugs under study. As (+)--.naltrexone is in preclinical development aiming at human clinical trials, this is especially exciting to have a blood brain barrier permeable, highly selective TLR4 antagonist that would be orally available, stable at room temperature, and appropriate for use from front lines through long--.term use. As Xalud Therapeutics received $2.7 million from the Army to move (+)--.naltrexone toward FDA Investigational New Drug status, it will be ready for entry into human clinical trials near term. Given our ongoing results, this would be a spectacular step forward for treating warfighters and veterans alike for drug abuse indications.

15. SUBJECT TERMS
toll like receptor 4 (TLR4); TLR4 agonists *non*-opioid (+)-naloxone and (+)-naltrexone; drug abuse; glial activation; therapeutic approach to treating drug abuse; opioids; cocaine

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Introduction:

The reinforcing and addictive properties of abused drugs, such as morphine and cocaine, are largely attributed to their ability to activate the mesolimbic dopamine pathway, resulting in increased extracellular dopamine in the nucleus accumbens shell (NAc). Under normal circumstances, the ventral tegmental area (VTA) strictly regulates dopamine levels within the NAc. Morphine and cocaine are known to interact with the central nervous system to produce distinctly different effects, both subjectively and physiologically; yet each drug is capable of increasing extracellular dopamine. To date, the bulk of research efforts have focused on how each drug interacts with its respective receptor targets on neurons. However, recently there has been more attention paid to the glial cells of the brain and how they might be involved in neurobiological mechanisms underlying the effects of drugs of abuse.

Morphine is known to act at mu-opioid receptors, which are located on neurons, both to produce its analgesic and rewarding/reinforcing effects. However, opioids—whether through prescription-based use to control pain or in abuse/illicit settings—have many unwanted side-effects, including tolerance (both for reward and pain relief), addiction, and severe withdrawal symptoms, among many others. Our laboratory recently published data demonstrating that morphine exerts many of these effects through activation of glial cells. Morphine-induced glial activation results in a powerful pro-inflammatory cascade, including the release of pro-inflammatory cytokines such as IL-1β and TNFα. These cytokines, and other pro-inflammatory molecules are neuroexcitatory and have the ability to interact with and effect neuronal functioning. Furthermore, we identified that the receptor through which morphine was inducing glial activation is Toll-Like Receptor 4 (TLR4), an innate immune receptor responsible for detecting pathogens. After showing that blockade of the TLR4 receptor improved morphine's analgesic properties and attenuated analgesic tolerance, we began to investigate the roll TLR4 signaling might have on morphine reinforcement. Preliminary studies demonstrated that systemic antagonism of TLR4 resulted in a blockade of both conditioned place preference (CPP) and self-administration, as well as a suppression of morphine-induced DA increase in the NAc. As intriguing as this finding is, it offers very little insight as to whether or not TLR4 signaling is directly involved in the mesolimbic pathway response to opioids, or whether there is some other less selective explanation for this phenomenon. Current pharmacological treatments for opioid addiction/abuse tend to be only effective and helping with decrease of illicit use, but require the continued use of a maintenance opioid, with lower abuse potential, that is costly and limited in success. Considering the increasing reports of opioid abuse, particularly abuse of prescription opioids, investigation into other treatment targets is of particular interest. TLR4 is an extremely interesting target to investigate, as early studies indicate that blockade of this receptor seems to preserve the desired effects of opioids (pain-relief) while diminishing unwanted effects (analgesic tolerance and reward/reinforcement leading to addiction/abuse).

Morphine is thought to exert most of its mesolimbic dopamine effects through actions in the VTA, where it disinhibits, or "turns down", VTA control of dopaminergic projections, allowing for more dopamine release in the NAc. However, the prevailing hypothesis is that cocaine induces an increase of dopamine in the NAc through blockade of dopamine transporters (DAT), re-uptake and clearance of dopamine from the synapse, resulting in an increased concentration of dopamine. In particular, research has focused on cocaine blockade of DAT in the NAc. However, medication development approaches focusing on disrupting inhibition of DAT by cocaine are largely unsuccessful, and cocaine abuse remains widespread, highly problematic, and extremely difficult to treat. We have recently demonstrated that cocaine also interacts with the Toll-Like Receptor 4 (TLR4) complex and that this interaction may be an important contribution to the neurobiological effects of cocaine underlying reinforcement, leading to subsequent abuse and addiction. Systemic interruption of TLR4-cocaine signaling results in a blockade of models of drug reinforcement including cocaine-induced dopamine increases in the NAc, and a suppression of

cocaine conditioned place preference, self-administration, and reinstatement to self-administration. These findings suggest that TLR4 signaling may be critical to both the reinforcing effects of cocaine and opioids such as morphine.

The purpose of this grant is to further investigate this remarkable finding to better understand the role of TLR4 signaling in drug reward/reinforcement in order to determine the potential clinical utility of this previously unknown mechanism. These results not only fundamentally alter and expand current understanding of the neurobiological mechanisms underlying drug reinforcement, but also offer a new potential target for medication development to treat cocaine abuse.

Body:

Task 1: **Obtain approval from the Institutional Animal Care and Use Committee at University of Colorado Boulder for work to be done in the Watkins-Maier lab (University of Colorado-Boulder), Bachtell lab (University of Colorado-Boulder) and Katz lab (National Institute on Drug Abuse Intramural Research Program).**

Task 1 has been completed on time for all sites and animal research is in progress.

Task 2: **Receive (+)-naltrexone, as needed across the project period, from Dr. Kenner Rice (National Institute on Drug Abuse Intramural Research Program).**

Task 2 is successfully undertaken; all (+)-naltrexone needed to date has been received by all research sites as committed by Dr. Kenner Rice

With accomplishment of Tasks 1 and 2, Milestone 1 was successfully achieved.

Watkins-Maier Research Lab:

Task 3 Aim 1A: Is morphine or cocaine CPP blocked by microinjecting a TLR4 antagonist (LPS-RS) into VTA or NAc shell?

Task 3 is in progress in the Watkins-Maier lab.

We have evidence indicating that cocaine interacts with and activates the TLR4-complex. Further, we have shown that cocaine interaction with TLR4 is an important modulator of drug-induced disruptions of the mesolimbic dopamine reward pathway that are thought to underlie the eurphoric effects of drugs, leading to drug abuse and addiction (see Task 4 for supporting data). In order to investigate whether these neurochemical findings are relevant to drug reward behavior, we are in the process of conducting CPP studies to test whether TLR4 antagonism within relevant brain regions of the mesolimbic dopamine pathway will correspondingly attenuate behavioral measures of drug reward. Previously, we had established ideal coordinates for bilateral VTA cannula placements. We have now identified appropriate microinjection drug dosing, timing and techniques that will not interfere the CPP testing paradigm, which is considered to be a highly valid model of drug reward but is also well known to be sensitive to disruptions. In the original timeline, CPP studies were proposed to precede the microdialysis studies outlined as Task 4 Aim 1B (below). However, given that data from those microdialysis studies should streamline the development of exact scientific design (for example,

physiologically and neurochemically relevant drug dosing) for all the sub-aims, allowing all tasks to be

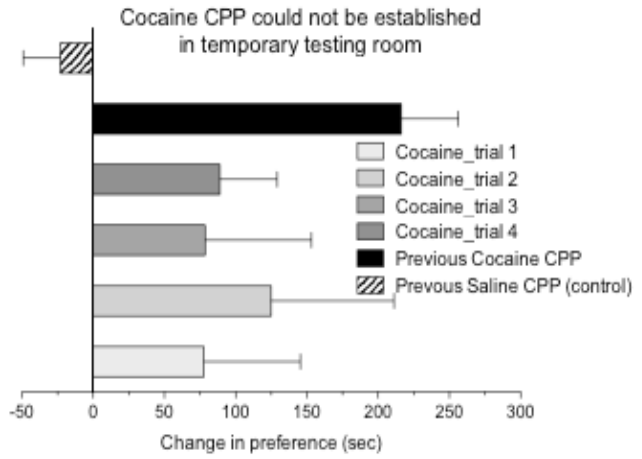


Figure 1: In order to examine whether we could successfully run our CPP paradigm in a new, temporary testing room (due to displacement from our established CPP testing room because of the 500 year flood causing severe damage needing months of repairs), we first tested whether naïve rats (without surgery to implant cannula) would demonstrate cocaine-induced place preference. Rats were pre-exposed to the testing apparatus to establish baseline preference and then assigned to treatment conditions in a counter-balanced fashion. Conditioning took place twice a day for 3 days, once in the morning and once in the afternoon with alternating treatments so that rats received 3 drug-paired and 3-vehicle paired conditioning sessions. The day after the last condioning session, rats were placed back into the boxes and place preference was assessed by calculated the difference between time spent in the drug-paired compartment of the CPP apparatus during place preference testing compared to pre-exposure. Over several trials it became evident that rats were not conditioning to cocaine, most likely due to the more disruptive nature of our temporary location.

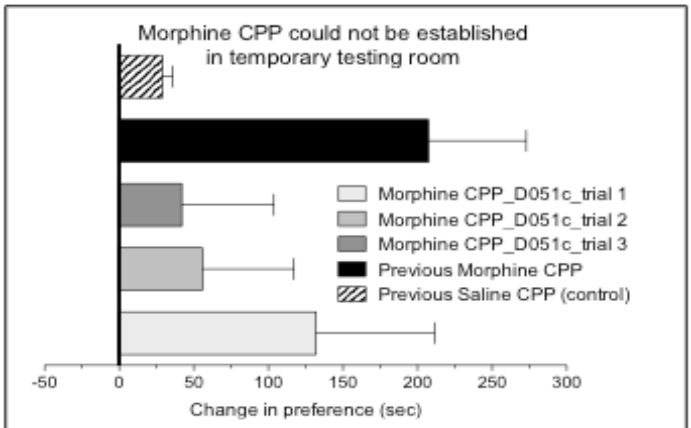


Figure 2: Because morphine tends to elicit different behavioral consequences compared to cocaine (for example, cocaine causes psychomotor activation along with it's euphoric effects whereas morphine, being a CNS depressant, is more likely to cause a sedative effect) that might increase tolerance to disruptive stimuli. The experiments were conducted as described in figure 1, except that conditioning took place once per day over 8 days, and each session lasted 45 minutes long. Again, as demonstrated by the graph, we were unable to get reliable morphine CPP.

completed in a more timely, efficient manner, we proceeded with the Task 4 studies. As predicted, the data from Task 4 Aim 1B (described below) was extremely accurate in establishing drug dosing and microinjection techniques such that we have now established appropriate LPS-RS dosing and microinjection techniques for CPP studies in Task 3.

In the early stages of running the CPP microinjection experiments outlined in Task 3, the 500 year flood and 1000 year rain that took place in Colorado in September of 2013, damaged and destroyed many buildings in the Boulder area. Unfortunately, our CPP testing room and corresponding animal colony were in one of those areas that was seriously damaged by the flood and backed up sewer system in the rooms. Not only were the animals currently undergoing CPP testing lost, but the rooms were so badly damaged that all animals and equipment had to be moved out immediately. The needed repairs were so extensive that early predictions of only 2-3 months were extended to more than 6 months to conduct the repairs and bring all elements up to IACUC/OLAW code. In the meantime, we attempted to set up the CPP testing paradigm in a temporary space. CPP is an easily disrupted paradigm, as the testing room must be absolutely protected from noise, odors, and other stimuli that might distract the rats or interfere with their conditioning/testing. We ran several series of experiments trying to re-establish our CPP effect with no success (**FIG 1** and **FIG 2**). Rats were unable to predictably learn to associate drug euphoria, from either cocaine (**FIG 1**) or morphine (**FIG 2**), with the correct corresponding compartment in the apparatus. We believe this is due to the comparative increase in activity (nearby testing, surgery, and wet lab rooms, increased traffic, equipment noise, etc.) in the area of the temporary testing space and due to ultrasonic vocalizations of rats in chronic pain experiments in nearby testing rooms (ultrasonic vocalizations readily pass through this building's walls). The failed CPP attempts in the temporary testing room, shown in figures 1 and 2, include data previously generated from our established CPP for comparison.

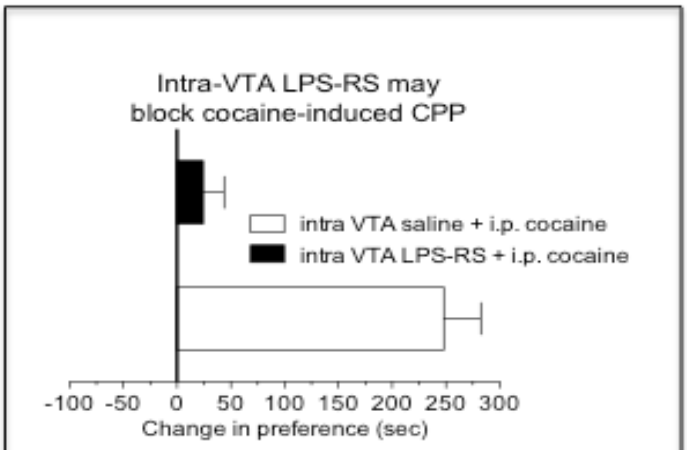


Figure 3 Rats were surgically implanted with bilateral microinjection cannula into the ventral tegmental area. Following a 7-day recovery period, rats were pre-exposed to the CPP testing apparatus to determine baseline preferences and ensure no pre-existing biases for any one compartment. Following pre-exposure, rats were assigned to treatments and testing conditions in a counter-balanced fashion. Then rats were conditioned once a day for 6 days, 30 minutes each session, with 3 days of drug-paired conditioning alternated with 3 days of vehicle-paired testing. Rats received intra-VTA microinjections of either 1µL LPS-RS (5µg) or saline and 10 minutes later received intraperitoneal cocaine (10mg/kg). Although the n per group (n= 3-4) is not yet high enough to allow for statistical analysis, these data strongly indicate that intra-VTA saline has no effect on cocaine-induced CPP and that 5 µg intra-VTA LPS-RS may attenuate cocaine-induced CPP. Statistical analysis will be conducted when the other required treatment groups (intra VTA LPS-RS + i.p. saline, intra VTA saline + i.p. saline) have been added and when n per group is a minimum of 6 rats).

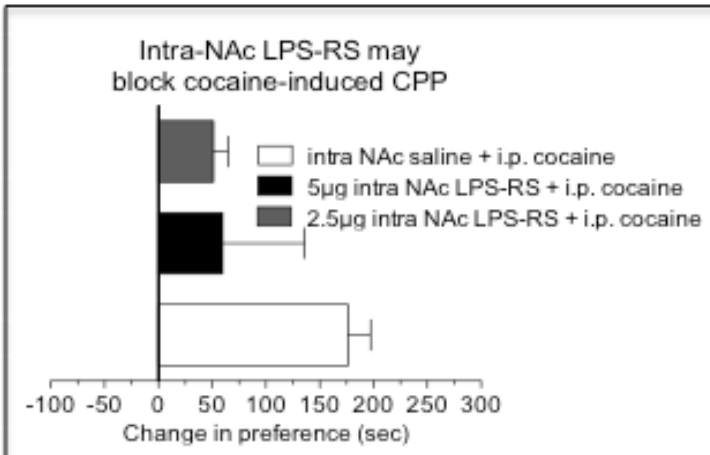


Figure 4: Rats were surgically implanted with bilateral microinjection cannula into the nucleus accumbens. The remainder of the experiment was conducted as described in figure 3, except that rats received intra NAc microinjections of 1 µL LPS-RS (2.5 or 5µg) or saline and 10 minutes later received intraperitoneal cocaine (10mg/kg). Although the n per group (n= 3-4) is not yet high enough to allow for statistical analysis, these data suggest that intra-VTA saline has no effect on cocaine-induced CPP and that 2.5 µg intra-VTA LPS-RA may attenuate cocaine-induced CPP. Statistical analysis will be conducted when the other required treatment groups (intra VTA LPS-RS + i.p. saline, intra VTA saline + i.p. saline) have been added and when n per group is a minimum of 6 rats).

In latter July we received permission to move our CPP equipment back into our original testing space and to house animals in the adjoining colony room. We have since have been able to re-establish our CPP phenomenon. Further, we have preliminary data for all drug groups of the studies proposed in Task 4 Aim1A. We have now established that our cannulation coordinates do not disrupt CPP testing. Preliminary data indicate that intra VTA- LPS-RS will suppress cocaine- and morphine-induced CPP (**FIG 3** and fig. 5, respectively). In addition, data gathered to date suggest that intra NAc LPS-RS may attenuate cocaine-induced CPP (**FIG 4**). Currently, for each study, we have an n=3-4. Although this data is promising, we do not yet have an appropriate n per group to conduct statistical analysis. Experiments to increase the n of each group are in progress. Studies to explore whether intra-NAc LPS-RS will alter morphine-induced CPP are planned to begin in the near future.

<u>Task 4 Aim 1B:</u> Are cocaine-induced increases in extracellular DA in NAc shell blocked by microinjection of LPS-RS into the VTA or NAc shell?

Task 4 is near completion in the Watkins-Maier lab.

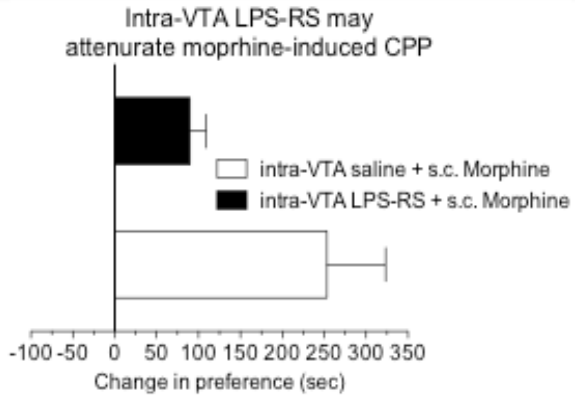


Figure 5: Rats were surgically implanted with bilateral microinjection cannula into the ventral tegmental area. The remainder of the experiment was conducted as described in figure 3, except that rats were conditioned once a day for 8 days, 45 minutes each session, with 4 days of drug-paired conditioning alternated with 4 days of vehicle-paired testing. Rats received intra-VTA microinjections of either 1µL LPS-RS (5µg) or saline and 10 minutes later received subcutaneous morphine (6mg/kg). Although the n per group (n= 3-4) is not yet high enough to allow for statistical analysis, these data strongly indicate that intra-VTA saline has no effect on cocaine-induced CPP and that 5 µg intra-VTA LPS-RS may attenuate cocaine-induced CPP. Statistical analysis will be conducted when the other required treatment groups (intra VTA LPS-RS + i.p. saline, intra VTA saline + i.p. saline) have been added and when n per group is a minimum of 6 rats.

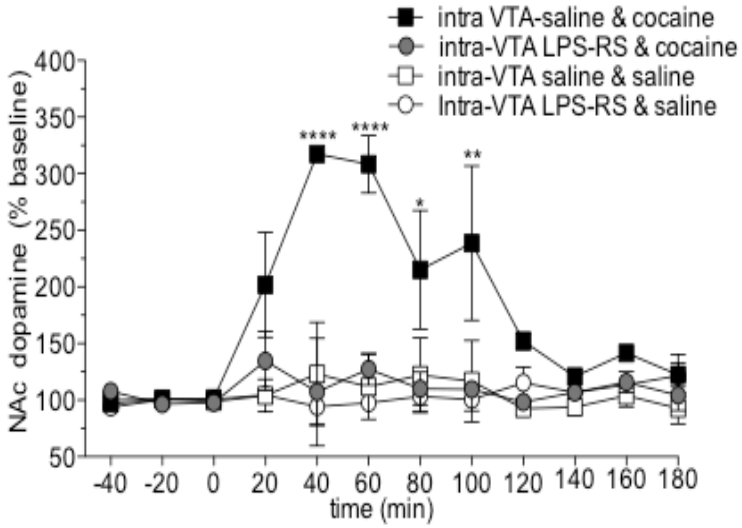


Figure 6: Rats were surgically implanted with microdialysis guide cannulae into the NAc shell and an ipsilateral microinjection cannula into the ventral tegmental area. Following a 7-day recovery period, rats were transported to the testing room for habituation, microdialysis probes were inserted and artificial cerebrospinal fluid flowed overnight. The next morning, after 3 baseline samples were collected, rats received either a 1uL injection of 5µg LPS-RS or 1µL sterile saline 10 minutes prior to an intraperitoneal (i.p.) injection of 10mg/kg cocaine or equivolume sterile saline. Samples were collected every 20 min for the duration of the study. Samples were analyzed using High Performance Liquid Chromatography (HPLC) to quantify dopamine concentrations. Analysis by repeated measures two-way ANOVA revealed a statistically significant interaction ($p<0.0001$) of time and treatment. Bonferroni post-hoc tests reveal that systemic cocaine administration produces elevated extracellular dopamine in the NAc that sustains for 40-100 min (****$p < 0.0001$, **$p < 0.01$, *$p <0.05$) following drug administration. Intra-VTA LPS-RS blocked this effect. There were no differences between other treatment groups. Data are means ± SEMs; n = 6/group.

We have evidence that cocaine interacts with TLR4, a previously unidentified cocaine target in the central nervous system. However, it was unknown whether or not cocaine interactions with TLR4 were relevant to the reward processing regions of the brain, particularly the mesolimbic dopamine pathway, known to critically underlie the rewarding effects of cocaine. These microdialysis studies were undertaken in order to further investigate the potential involvement of these brain regions, namely the dopaminergic projections from the VTA terminating in the NAc. It is well established that drugs of abuse, such as morphine and cocaine, increase dopamine levels in the NAc and these increased dopamine levels correspond to the subjective experience of drug reward. The data we have gathered to-date is compelling, suggesting that TLR4 activation is such a critical component of mesolimbic pathway activation that when TLR4-signalling is blocked, dopamine levels remain at baseline even in the presence of systemic cocaine or morphine. Interestingly, we have found intra-VTA blockade of TLR4 signing is particularly critical for morphine- or cocaine-induced increases of dopamine within the NAc. Intra-VTA microinjections of the TLR4 antagonist LPS-RS, suppress cocaine-induced as well as morphine induced increases of dopamine within the NAc (**FIG 6** and **FIG 7**, respectively).

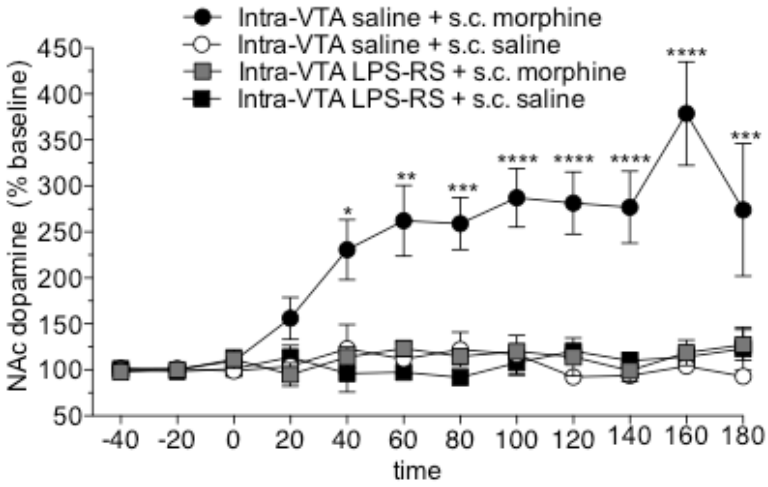


Figure 7: Experiment was conducted as described in figure 6, except that rats received either an intra VTA 1uL injection of 5µg LPS-RS or 1µL sterile saline immediately prior to an subcutaneous injection of 6mg/kg morphine or equivolume sterile saline. Samples were collected every 20 min for the duration of the study. Samples were analyzed using High Performance Liquid Chromatography (HPLC) to quantify dopamine concentrations. Analysis by repeated measures two-way ANOVA revealed a statistically significant interaction ($p<0.0001$) of time and treatment. Bonferroni post-hoc tests reveal that systemic morphine administration produces elevated extracellular dopamine in the NAc that sustains for 40-180 min (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$) following drug administration. Intra-VTA LPS-RS blocked this effect. There were no differences between other treatment groups. Data are means ± SEMs; n = 6/group.

Because of the complete blockade of drug-induced dopamine increases observed with intra-VTA administration of LPS-RS, in order to ensure that intra-VTA LPS-RS administration wasn't just broadly "turning off" or interfering with mesolimbic dopamine pathway functioning, we conducted a control study. In this case, rats received intra VTA injections LPS-RS following by a microinjection of the endogenous peptide, neurotensin. Neurotensin has been shown to increase intra-NAc dopamine concentrations when injected into the VTA; given that neurotensin is endogenous in origin, it is very unlikely to interact with the TLR4 complex. Our results indicated that neurotensin-induced increases of NAc dopamine are preserved in the presence of intra-VTA LPS-RS and therefore it is unlikely that LPS-RS is

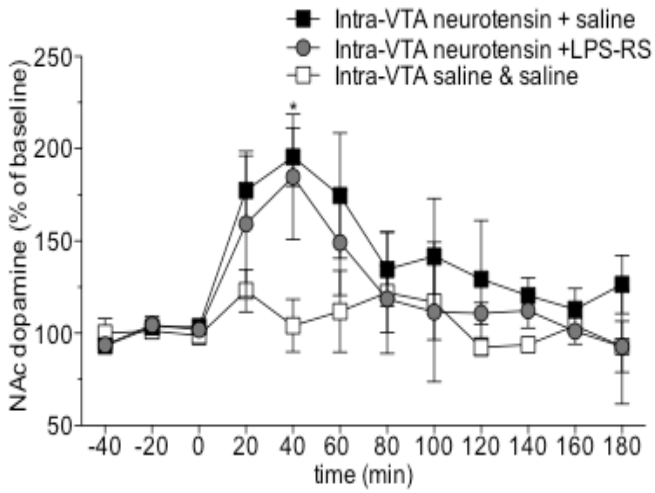


Figure 8: Experiment was conducted as described in figure 6, except that rats received a 10nM intra-VTA injection of neurotensin 10 minutes following a 1uL intra-VTA microinjection of 5µg LPS-RS. Analysis by repeated measures ANOVA indicate that there is a significant effect of treatment ($p = 0.002$). Bonferroni post-hoc tests reveal that intra-VTA administration of neurotensin elicits increased extracellular levels of dopamine in the nucleus accumbens shell ($p < 0.05$) and is unaffected by intra-VTA administration of LPS-RS.

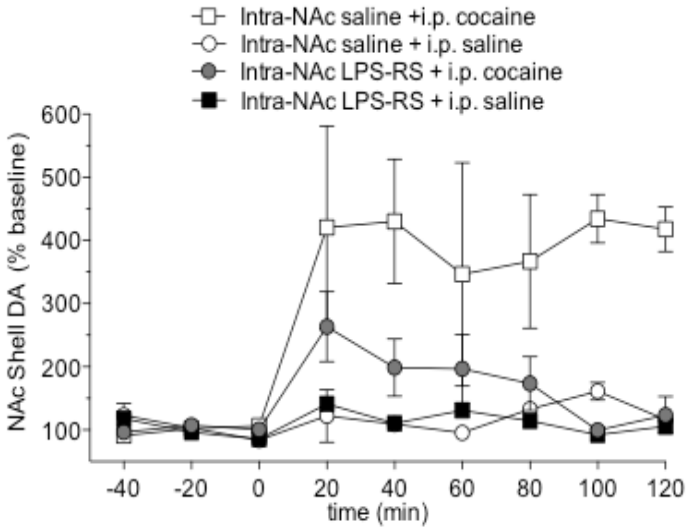


Figure 9: Study was conducted as previously described in figure 6, with the exception that a SciPro omni-probe guide cannula, allowing for both microinjections and microdialysis in the same brain region, was implanted and aimed at the NAc shell. During the experiment, rats received 2.5 µg intra-NAc LPS-RS in 0.5 µL and 10 minutes later received an interperitoneal injection of 10mg/kg cocaine. These data have not yet been analyzed because of low group n (n=3-4) but are thus far very promising and suggest that intra-NAc LPS-RS may attenuate cocaine induced dopamine increase in the NAc.

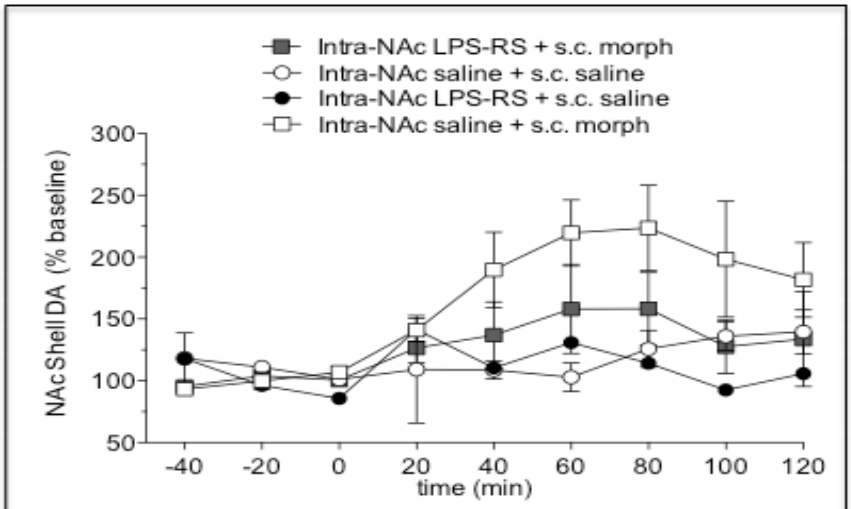


Figure 10: Study was conducted as previously described in figure 6. During the experiment, rats received 2.5 µg intra-NAc LPS-RS in 0.5 µL and then immediately received a subcutaneous injection of 6mg/kg morphine. These data have not yet been analyzed because of low group n (n=3-4) but suggest that intra-NAc LPS-RS may attenuate morphine-induced dopamine increase in the NAc.

ubiquitously inhibiting the mesolimbic dopamine pathway (**FIG 8**).

Studies investigating the effect of TLR4 signaling blockade within the NAc are currently underway. Preliminary data indicate that LPS-RS microinjected into the NAc may attenuate cocaine- and morphine-induced dopamine increases within the NAc (**FIG 9** and **FIG 10**). Interestingly, to date these data suggest that TLR4 signaling in the NAc may not be as critical a mechanism underlying drug induced alterations of the mesolimbic dopamine pathway. However, as currently n = 2-4 per group, we cannot yet draw any firm conclusions. Studies to increase the n of each group are underway.

Task 5 Aim 3A&B: Which cell types(s) (microglia, astrocytes, oligodendrocytes, endothelial cels, neurons) express TLR4 in VTA and/or NAc, basally vs. after chronic morphine/cocaine?

These studies are ongoing.

As discussed in our previous report, through western blot screening, we have discovered that commercially available TLR4 antibodies are not selective; that is, there they exhibit significant non-specific binding which makes their use in immunohistochemistry suspect. However, the TLR4 signaling complex is also comprised of an MD-2 co-factor, which is an integral component of cocaine- and morphine- induced TLR4 activation. We found an MD-2 antibody that western blots indicated was promising in its selectivity. We have since developed and fine-tuned the immunohistochemistry protocol to ensure clean staining in brain tissues with good signal to noise.

We conducted a preliminary study to examine whether or not there would be MD-2 expression in naïve rat brains compared to the brains of rats that had activated TLR4 signaling. In order to achieve this, those rats received a dose of the classical TLR4 antagonist, lipopolysaccharide (LPS) that our laboratory has documented repeatedly to induced robust TLR4 signaling, that is detectable for one to four hours following injection.

We encountered an interesting and relevant finding that there was more MD-2 staining in naïve brains of rats compared to the brains from rats that had received LPS. This may suggest that antibody binding is disrupted by LPS binding. This indicates that even while examining brains from rats that have been on chronic cocaine or morphine, it may be most advantageous to remove tissue once the drug has been metabolized for identify available MD-2 and infer whether there is a long-lasting upregulation of TLR4 complex expression, possibly leading to increased TLR4 proinflammatory signaling, or not.

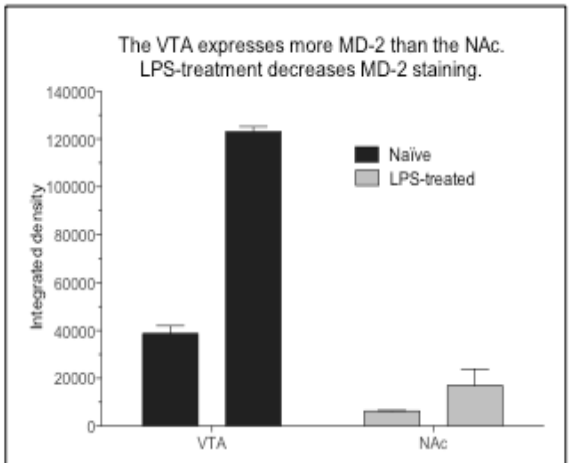


Figure 2: Immunihistochestry to label MD-2 in the NAc and the VTA of brains from naïve rats compared to those treated with LPS 90 minutes prior to collection. The VTA appears to express much higher density of MD-2 than the NAc. Further, LPS administration appears to diminish the amount of available MD-2 for anti-body labeling. These data have not yet been statistically analyzed because of small group sizes (n=2-3); this task is in progress.

Bachtell Research Lab:

Task 3 Aim 2A. Self- administration: Is cocaine reinforcement inhibited by systemic dosing with the TLR4 signaling inhibitor (+)-naltrexone? If so, is cocaine reinforcement inhibited by microinjecting LPS-RS into VTA or NAc shell?

Experiment 2A1: Acquisition of Cocaine Self-Administration

Methods: This experiment assessed the direct effect of TLR4 receptor antagonism on acquisition and maintenance of cocaine self-administration. We also tested the indirect effects of TLR4 antagonism on subsequent cue- and cocaine-induced reinstatement in the animals run to date. Osmotic minipumps (14-day 2mL) were filled with either (+)-naltrexone (15 mg/kg) or sterile water and implanted two days prior to the start of self-administration. Animals were then permitted to self-administered cocaine (0.5 mg/kg/infusion, iv) two hours per day over the next 17 days. After the self-administration session on day 13, the minipumps were removed and the animals continued with cocaine self-administration for an additional 4 days. The animals then underwent six days of extinction training followed by cue and then cocaine-induced reinstatement (15mg/kg, ip), where they were allowed to lever-press for two hours.

Results: Chronic administration of 15 mg/kg/day (+)-naltrexone revealed no change in the acquisition or maintenance of intravenous cocaine self-administration without prior lever-press training. Thus, (+)-naltrexone failed to affect the acquisition of self-administration during the first week of testing, and cocaine intake stabilized at similar levels in all groups during the second week of self-administration. After the last self-administration session, rats were progressed to extinction conditions to identify the indirect effects of (+)-naltrexone administration on cocaine seeking. Animals administered (+)-naltrexone during cocaine self-administration exhibited significant reduction in drug-paired lever responding compared to controls during the first extinction test. These data suggest that TLR4 inhibition during cocaine intake may decrease subsequent drug seeking that is indicative of drug craving. Following extinction, discrete cues that previously were paired with cocaine injections showed similar abilities to reinstate cocaine seeking in both (+)-naltrexone and control groups. Likewise, the administration of 15 mg/kg cocaine produced reinstatement to cocaine seeking similarly in both groups as well. Together, these data suggest that TLR4 inhibition with (+)-naltrexone administration during cocaine self-administration does not affect acquisition and maintenance of cocaine intake, but may reduce subsequent cocaine seeking. Studies in the upcoming year will assess whether increased doses of (+)-naltrexone effectively reduce the acquisition and maintenance of cocaine self-administration.

Experiment 2A2: Effects of (+)-Naltrexone on Cocaine Reinforcement

Methods: This experiment assessed the effects of TLR4 antagonisms on cocaine reinforcement using a progressive ratio schedule. The animals were allowed to self-administer cocaine (0.5 mg/kg/infusion, iv) for two hours per day on a fixed ratio 1 (FR1) schedule for six days, and were then moved to a fixed ratio 5 (FR5) schedule for four days. Osmotic minipumps (7-day 2mL) were filled with either (+)-naltrexone (15 mg/kg) or sterile water and implanted after the last day of self-administration on FR5 (one day prior to the start of the progressive ratio schedule). The animals self-administered cocaine on progressive ratio for five days, and the pumps were removed following the final self-administration session. The animals then underwent nine days of extinction training followed by two-hour cue and cocaine-induced reinstatement (15mg/kg, ip) sessions.

Results: The progressive ratio schedule of reinforcement is the hallmark procedure used to identify the reinforcing efficacy of drugs of abuse by assessing the amount of effort an animal is willing to exert to obtain cocaine reinforcement. Chronic administration of 15 mg/kg/day (+)naltrexone during progressive ratio testing produced no change in either the number of cocaine infusions delivered or the final ratio

completed (breakpoint) to earn a cocaine infusion. These findings suggest that 15 mg/kg/day (+)naltrexone does not influence cocaine reinforcement mechanism. Future work will assess the effects of 30 mg/kg/day (+)naltrexone on progressive ratio responding.

Task 4. Aim 2B. Is cocaine reinstatement to drug seeking blocked by systemic (+)-naltrexone? If so, is cocaine-induced reinstatement of drug seeking inhibited by LPS-RS microinjection into the VTA or NAc shell?

Experiment 2B1: Effects of Systemic (+)-Naltrexone on Cocaine reinstatement

Methods: This experiment assessed the effect of acute administration of (+)-naltrexone on cocaine-induced reinstatement. The animals self-administered cocaine (0.5 mg/kg/infusion, iv) for two hours per day for fifteen days. They then underwent extinction training for five days where lever presses were not reinforced. During reinstatement testing, the animals first had a two-hour extinction session immediately followed by a pretreatment of two injections of (+)-naltrexone (0-15 mg/kg, sc) or saline vehicle spaced thirty minutes apart. After the second (+)-naltrexone injection, the animals received either a cocaine (15 mg/kg, ip) or saline vehicle prime. Non-reinforced lever pressing (active and inactive) was recorded during the two-hour session.

Results: All animals were trained to self-administer cocaine over 3 weeks. Lever responding was then extinguished in daily sessions where lever responding no longer produced the delivery of a cocaine infusion. After lever responding was extinguished to criterion, responding was reinstated by the administration of 15 mg/kg cocaine preceded by an acute (+)-naltrexone (7.5 or 15 mg/kg, ip) or vehicle injection. An acute treatment of cocaine produced robust reinstatement of cocaine seeking that was significantly reduced by the pretreatment of 15 mg/kg (+)-naltrexone (**Fig 11**). These results suggest that inhibition of TLR4 with (+)-naltrexone is sufficient to inhibit cocaine seeking.

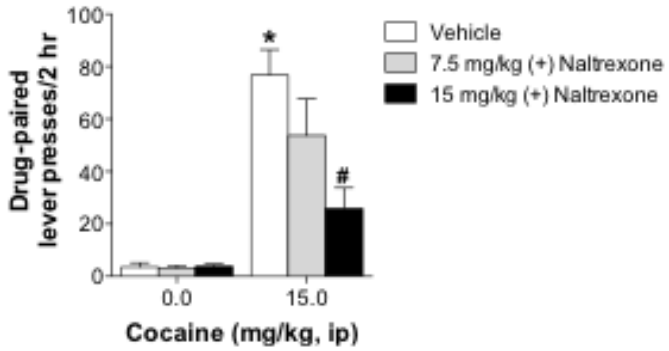


Figure 11. Acute administration of (+)-naltrexone dose-dependently reduced cocaine-primed reinstatement. * Signficant from Vehicle/Vehicle, $p < 0.05$; # Signficicant from Vehicle/15 mg/kg Cocaine, n = 10-12/group

Experiment 2B2: Effects of LPS-RS microinjections on Cocaine Reinstatement

Methods: These experiments are designed to assess the effect of TLR4 antagonism specifically in the nucleus accumbens shell (NAcSh) or ventral tegmental area (VTA) on cocaine-induced reinstatement. Prior to the start of self-administration, the animals were implanted with both an intravenous catheter and guide cannula directed into either the NAcSh or VTA. After recovery from surgery, the animals self-administered cocaine (0.5 mg/kg/infusion, iv) for two hours per day for fifteen days. Animals then underwent extinction training in daily two-hour extinction sessions for nine days. During reinstatement testing, the animals first had a two-hour extinction session immediately followed by a microinjection pre-treatment of LPS-RS (5 µg/side) or saline vehicle followed by a cocaine (15 mg/kg, i.p.) or saline vehicle prime. Non-reinforced lever pressing (active and inactive) was recorded during the two-hour session.

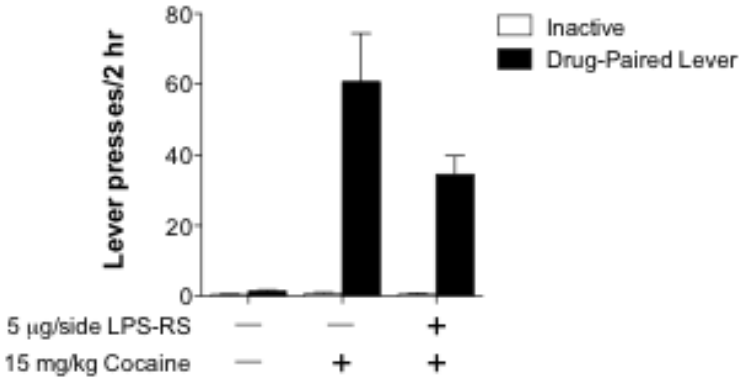


Figure 12. Acute administration of LPS-RS into the ventral tegmental area (VTA) produced a trend toward reduced cocaine-primed reinstatement. n = 5-8/group)

Results: These experiments are underway and we have preliminary data supportive of our hypotheses. Thus far, we have only tested the effects of TLR4 antagonism in the VTA. In this experiments, all animals were trained to self-administer cocaine over 3 weeks and lever responding was then extinguished in daily sessions where lever responding no longer produced the delivery of a cocaine infusion. After lever responding was extinguished to criterion, responding was reinstated by the systemic administration of 15 mg/kg cocaine preceded by an acute intra-cranial administration of 5 µg/side LPS-RS or vehicle into the VTA. Cocaine induced significant reinstatement of cocaine seeking that was reduced by the pretreatment of LPS-RS into the VTA (**Fig. 12**). These results suggest that local inhibition of TLR4 in the VTA is associated with cocaine relapse is sufficient to inhibit cocaine seeking. This study is currently incomplete and we intend to increase the sample sizes to attain statistical significance. In addition, we intend to explore the role of TLR4 in the NAcSh by conducting analogous experiments with LPS-RS administered directly into the NAcSh.

Katz Research Lab:

Dr. Takato Hiranita left our employ on 8/7/2013. He was replaced by Dr. Zachary Hurwitz who was with us from 7/29/13 to 3/1/2014. Dr. Hurwitz turned out to be a poor choice to replace Dr. Hiranita. During the short time that he was with us he learned the catheterization surgery and the fundamental techniques for training subjects to self-administer drugs. However just as we were prepared to initiate studies, Dr. Hurwitz left our employ for another position.

Dr. Claudio Zanettini joined us in late May of this year. We selected him from among about six final candidates because he had experience catheterizing subjects. Dr. Zanettini has been employed previously in well-known laboratories in the field of drug abuse and has the skills to successfully conduct and move the project forward (see CV in attachment). A detailed description of Dr. Zanettini activities since his start in May can be found in the Table below.

Dates		Activity
From	To	
5/19/2014	6/2/2014	Arrival and NIDA standard training for safe conduct of laboratory studies and animal care/welfare
6/3/2014	6/10/2014	Orientation to self-administration equipment in the NIDA labs, tested and modified self-administration program, ordered drugs.
6/10/2014	6/17/2014	Rat catheter implantation in jugular vein for self-administration

6/18/2014	6/29/2014	Post-surgery care of subjects, defined some technical details of the experiment
6/30/2014	7/7/2014	Rat self-administration of 0.1 mg/kg/inj heroin
7/8/2014	7/15/2014	Rat self-administration of 0.056 mg/kg/inj heroin
7/16/2014	8/6/2014	Extinction phase of the experiment and catheter implantation in 3 new subjects
8/7/2014	9/11/2014	Reinstatement phase of the experiment and catheter implantation in 4 new subjects
9/11/2014	9/21/2014	Rat self-administration of 0.1 mg/kg/inj heroin in newly implanted subjects and catheter implantation of 5 additional subjects
9/21/2014	Ongoing	Training for cannula implantations in VTA and NAc shell, infusions, and histological verification of placements with Dr. Gianluigi Tanda

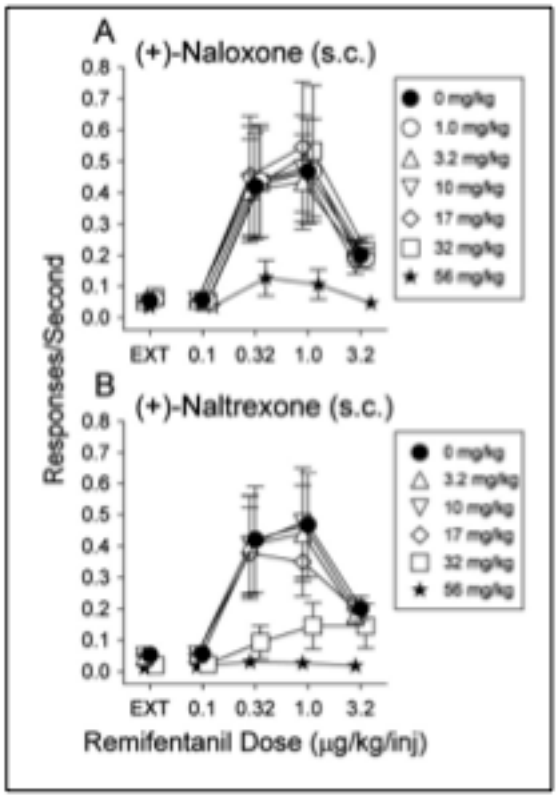
Task 3. Aim 1B. Are morphine-induced increases in extracellular DA in NAc shell blocked by microinjection of LPS-RS into the VTA or NAc shell?

All of Aim 1B is being done in the Watkins lab. See above for data update.

Task 4. Aim 2A. Self-administration: Is morphine reinforcement inhibited by systemic dosing with the TLR4 signaling inhibitor (+)-naltrexone? If so, is morphine reinforcement inhibited by microinjecting LPS-RS into VTA or NAc shell?

Task 4. Aim 2A. Self-administration: Is morphine reinforcement inhibited by systemic dosing with the TLR4 signaling inhibitor (+)-naltrexone? If so, is morphine reinforcement inhibited by microinjecting LPS-RS into VTA or NAc shell?

The first part of this task (4b & 4c) has been completed and preliminary results were reported last year. Sprague-Dawley rats (Taconic Farms, Germantown, New York) weighing approximately 300 g at the start of the study, served as subjects. Subjects were acclimated to a temperature- and humidity-controlled vivarium for at least one week with a 12:12-h light:dark cycle (lights on at 07:00 hours) during which food (Scored Bacon Lover Treats, BIOSERV, Frenchtown, NJ) and tap water were available at all times. After acclimation, body weights were maintained at approximately 320 g by adjusting the daily food ration with water remaining available at all times in the home cages. Care of the subjects was in accordance with the guidelines of the National Institutes of Health and the National Institute on Drug Abuse Intramural Research Program Animal Care and Use Program, which is fully accredited by AAALAC International.



Subjects were surgically prepared under anesthesia (ketamine 60.0 mg/kg, i.p. and xylazine 12.0 mg/kg, i.p.) with a chronic indwelling catheter in the right external jugular vein. The catheter exited the subject at the mid-scapular region of its back. Catheters were infused daily with 0.1 ml of

a sterile saline solution containing heparin (30.0 IU/ml) and penicillin G potassium (250,000 IU/ml) to minimize the likelihood of infection and the formation of clots or fibroids. All animals were allowed to recover from surgery for approximately seven days before drug self-administration studies were initiated.

Experimental sessions were conducted daily with subjects placed in operant-conditioning chambers (modified ENV-203, Med Associates, St. Albans, VT) that measured 25.5 x 32.1 x 25.0 cm that were enclosed within sound-attenuating cubicles equipped with a fan for ventilation and white noise to mask extraneous sounds. On the front wall of each chamber were two response levers, 5.0 cm from the midline and 4.0 cm above the grid floor. A downward displacement of either lever with a force approximating 0.20 N defined a response, and always activated a relay mounted behind the front wall of the chamber producing an audible "feedback" click. Six light-emitting diodes (LEDs, three yellow and three green ones) were located in a row above each lever. A house light was located at 25 cm above the grid floor (near the ceiling) at the center of the front wall of the chamber. A receptacle for the delivery of 45-mg food pellets via a pellet dispenser (Med Associates, Model ENV-203-20), was mounted on the midline of the front wall between the two levers and 2.0 cm above the floor. A syringe infusion pump (Model 22, Harvard Apparatus, Holliston, MA) placed above each chamber delivered injections of specified volumes from a 10 ml syringe. The syringe was connected by Tygon tubing to a single-channel fluid swivel (375 Series Single Channel Swivels, Plymouth Meeting, PA) which was mounted on a balance arm above the chamber.

4b. Implant rats with indwelling jugular catheters, train on self-administration, pilot studies on (+)-naltrexone dose with dose adjustment as needed, test for effect of systemic (+)-naltrexone on morphine self-administration

4c. Unblinding of data and data analysis

Rats were first trained on cocaine self-administration and were subsequently tested with remifentanil substituted for cocaine. Remifentanil injections reliably maintained self-administration at high rates that were dependent on dose of drug. **FIG 13** (filled circles) shows the inverted U-shaped dose-effect curve for remifentanil; this shape of the dose-effect curve is characteristic of that for all drugs of abuse. Dose explorations (task 4b) indicated that the highest rate of responding was maintained at a remifentanil dose of 1.0 ug/kg/inj, with lower response rates at higher and lower doses (**FIG 13**, filled circles). Response rates were significantly ($F(4,20) = 4.20$, $p = 0.013$) affected by remifentanil dose, and post-hoc tests indicated that rates maintained by 1.0 µg/kg/inj of remifentanil were significantly greater than those obtained when responses had no consequences (EXT).

The effects of the TLR4 antagonists (+)-naloxone and (+)-naltrexone were tested on self-administration of the µ-opioid agonist, remifentanil. Remifentanil was chosen for testing rather than morphine due to its very short half-life which promotes high rates of self-administration and stability of lever pressing. The high rates of responding maintained promote an exceptional signal-to-noise ratio. Rates of responding when responses have no consequences (EXT) are much lower than the rates of responding maintained by the 1.0 ug/kg dose of remifentanil resulting in a S/N ratio for remifentanil (0.001 mg/kg/inj: 0.689; EXT: 0.0145) of 47.6. This enhanced signal-to-noise ratio increases the sensitivity of the procedure for the detection of antagonism. In contrast, the S/N ratio for heroin (0.01 mg/kg/inj: 0.0727; EXT: 0.0129) is 5.64; which is about one order of magnitude lower.

The pilot studies with different doses of (+)-naloxone (s.c.) administered immediately before the self-administration sessions indicated a dose-dependent suppression of remifentanil self-administration (**FIG 13A**). The maximally effective dose of (+)-naloxone was 56 mg/kg (compare filled circles to stars). A two-way repeated measures ANOVA indicated significant effects of remifentanil dose ($F(4,40) = 5.22$, $p = 0.005$) and the interaction of remifentanil and (+)-naloxone doses ($F(8,40) = 2.34$, $p = 0.036$). Post-hoc tests indicated that the effects of 56 mg/kg of (+)-naloxone significantly ($p \leq 0.012$) decreased response rates maintained by the 0.32 ($t = 2.99$) and 1.0 ($t = 2.98$) ug/kg/inj dose of remifentanil. Decreases in remifentanil self-administration were also obtained with i.p. injections of (+)-naloxone (data not shown).

The pilot studies with different doses of (+)-naltrexone (s.c.) immediately before the self-administration sessions also showed a dose-dependent suppression of remifentanil self-administration (**FIG 13B**, compare filled circles to open squares and stars). Decreases in remifentanil self-administration were obtained at a lower dose (32 mg/kg) of (+)-naltrexone compared with the effects of (+)-naloxone.

A second group of subjects was trained with food reinforcement in order to assess the specificity of the effects of (+)-naloxone on remifentanil self-administration. Experimental procedures were identical to those detailed above except that each completion of five responses delivered a food pellet rather than an injection of remifentanil. The selectivity of the effects of (+)-naloxone and (+)-naltrexone were assessed by comparing the effects at the maximal rates of responding maintained by either the dose of remifentanil or the amount of food that maintained the highest rate of responding. As shown in **FIG 14**, the decreases in food reinforced responding occurred at about the same doses as those that decreased responding maintained by remifentanil.

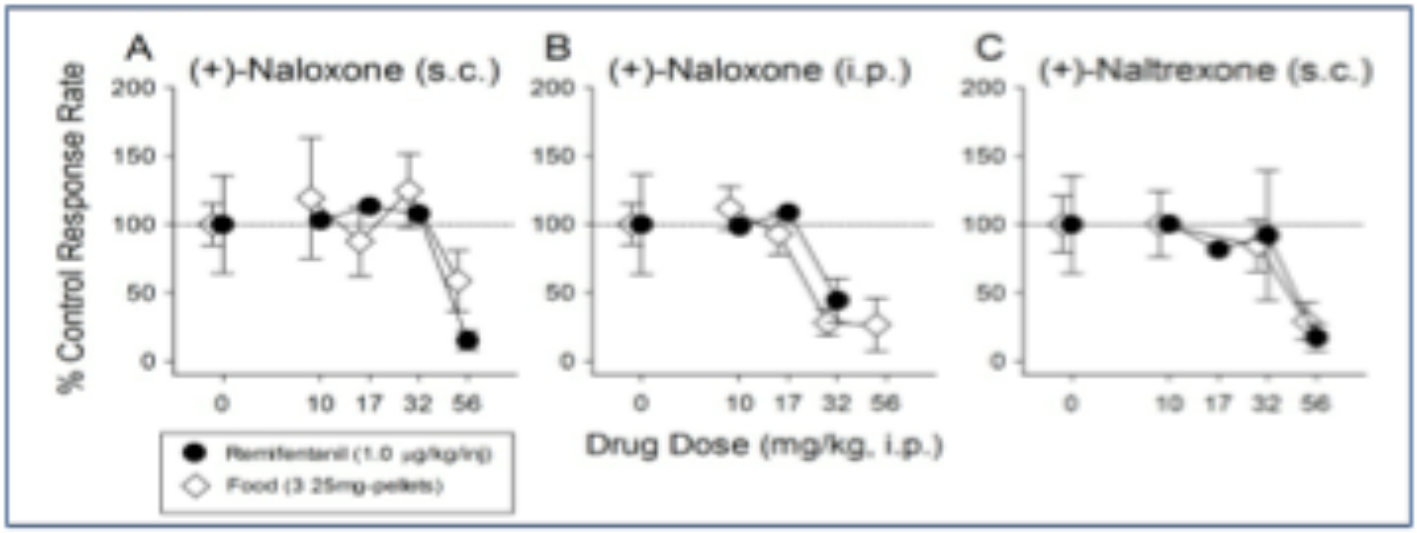


Figure 14: Effects of (+)-naloxone and (+)-naltrexone on responding maintained by injections of remifentanil or food presentation. Ordinates: response rates as a % of control rates of responding; abscissae: drug dose in mg/kg. EXT: Extinction. (+)-Naloxone was administered by the subcutaneous or intraperitoneal route at 5 min before sessions. (+)-Naltrexone was administered by the subcutaneous route at 5 min before sessions. Note that (+)-naloxone and (+)-naltrexone were equipotent in decreasing responding maintained by injections of remifentanil or food presentation. Data are means ± SEMs; n = 6/group.

4d. Cannula implantations in VTA and NAc shell, pilot studies to define doses, self-administration squads run, perfusion, histology to check cannula placements (after Aim 2B: task 5e), replacement of rats with poor cannula placements, site specificity studies as needed

4e. Unblinding of data and data analysis (timeframe: after full study of each brain site; completed in months 30-31)

Dr. Zanettini is working with Dr. Gianluigi Tanda (NIDA/IRP) to learn all techniques necessary for these experiments. Dr. Zanettini has experience with these techniques, so the amount of time necessary for this training is not anticipated to be large. He will be learning all necessary techniques for cannula implantations in VTA and NAc shell, delivery of infusions at a rate sufficiently low that tissue damage will not results, and techniques for histological verification of cannula placements.

Task 5. Aim 2B. Is morphine reinstatement to drug seeking blocked by systemic (+)-naltrexone? If so, is morphine-induced reinstatement of drug seeking inhibited by LPS-RS microinjection into the VTA or NAc shell?

Sprague-Dawley rats (Taconic Farms, Germantown, New York) weighing approximately 300 g at the start of the study, served as subjects. Subjects were acclimated to a temperature- and humidity-controlled vivarium for at least one week with a 12:12-h light:dark cycle (lights on at 07:00 hours) during which food (Scored Bacon Lover Treats, BIOSERV, Frenchtown, NJ) and tap water were available at all times. After acclimation, body weights were maintained at approximately 320 g by adjusting the daily food ration. Water was available at all times in the home cages. Care of the subjects was in accordance with the guidelines of the National Institutes of Health and the National Institute on Drug Abuse Intramural Research Program Animal Care and Use Program, which is fully accredited by AAALAC International.

Subjects were surgically prepared under anesthesia (ketamine 60.0 mg/kg, i.p. and xylazine 12.0 mg/kg, i.p.) with a chronic indwelling catheter in the right external jugular vein. The catheter exited the subject at the mid-scapular region of its back. Catheters were infused daily with 0.1 ml of a sterile saline solution containing heparin (30.0 IU/ml) and penicillin G potassium (250,000 IU/ml) to minimize the likelihood of infection and the formation of clots or fibroids. All animals were allowed to recover from surgery for approximately seven days before drug self-administration studies were initiated.

Experimental sessions were conducted daily with subjects placed in operant-conditioning chambers (modified ENV-203, Med Associates, St. Albans, VT) that measured 25.5 x 32.1 x 25.0 cm that were enclosed within sound-attenuating cubicles equipped with a fan for ventilation and white noise to mask extraneous sounds. On the front wall of each chamber were two response levers, 5.0 cm from the midline and 4.0 cm above the grid floor. A downward displacement of either lever with a force approximating 0.20 N defined a response, and always activated a relay mounted behind the front wall of the chamber producing an audible "feedback" click. Six light-emitting diodes (LEDs, three yellow and three green ones) were located in a row above each lever. A house light was located at 25 cm above the grid floor (near the ceiling) at the center of the front wall of the chamber. A receptacle for the delivery of 45-mg food pellets via a pellet dispenser (Med Associates, Model ENV-203-20), was mounted on the midline of the front wall between the two levers and 2.0 cm above the floor. A syringe infusion pump (Model 22, Harvard Apparatus, Holliston, MA) placed above each chamber delivered injections of specified volumes from a 10 ml syringe. The syringe was connected by Tygon tubing to a single-channel fluid swivel (375 Series Single Channel Swivels, Plymouth Meeting, PA) which was mounted on a balance arm above the chamber.

5b. Move rats from the systemic (+)-naltrexone study (Aim 2A, task 4b) into reinstatement paradigm, re-stabilize responding, drug withdrawal, test systemic (+)-naltrexone on drug cue (morphine)-induced drug seeking.

5c. Unblinding of data and data analysis

It proved impractical to move subjects from task 4b into this study so experimentally naïve subjects were used for reinstatement paradigm and testing with systemic (+)-naltrexone on drug cue (morphine)-induced drug seeking.

Procedures for the acquisition of self-administration generally followed those described by Bossert et al. (2012). Experimental sessions were comprised of an initial 10-sec timeout period in which all lights were off and responding had no programmed consequences, followed by a responding period of 3 hours. In the responding period, one response on the active lever (FR 1) turned off the LED array above the lever, produced a click of a feedback relay and a 2.3 sec activation of the infusion pump during which the houselights were extinguished. Responding on the inactive lever produced a feedback clicker but no other programmed consequences.

In Phases 1 (sessions 1-7) and 2 (sessions 8-14) of acquisition, each response produced, respectively, infusions of 0.1 and 0.056 mg/kg of heroin. Phase 3 (extinction) started on session 15 when

the coefficient of variation for number of infusions during the last 3 sessions was less than 0.3. Phase 3 extinction sessions were identical to those in the preceding phases with the exception that no syringe was placed in the infusion pump and therefore responding on the active lever did not produce injections but did produce the stimuli previously paired with the delivery of heroin. Phase 3 extinction lasted 15 sessions.

Numbers of responses on the active and inactive levers during acquisition and extinction are shown in **FIG 15**, and were consistent with previous published results (e.g. Bossert et al. 2012). During the first week (0.1 mg/kg/inj), responding on the active lever increased and reached levels above those on the inactive lever. Decreasing the unit dose to 0.056 mg/kg/inj increased active lever responses. At the end of Phase two the number of responses averaged 32 ± 7.3 responses per session (**FIG 15**). During extinction responding on both the active and inactive levers first increased and subsequently decreased to a plateau within about 6 sessions.

From session 30 subjects were tested for heroin-induced reinstatement of active lever responding. One of the aims of this initial part of this task was to establish doses of heroin that would reliably induce reinstatement for the later assessment of (+)-naltrexone antagonism. In the first and second series of tests the dose of heroin that produced the maximal reinstatement was 0.56 mg/kg. The amount of reinstatement decreased with each series of tests. These results indicate that the dose that will be most reliable in producing reinstatement is 0.56 mg/kg and that the reinstating effectiveness of heroin is diminished with each test.

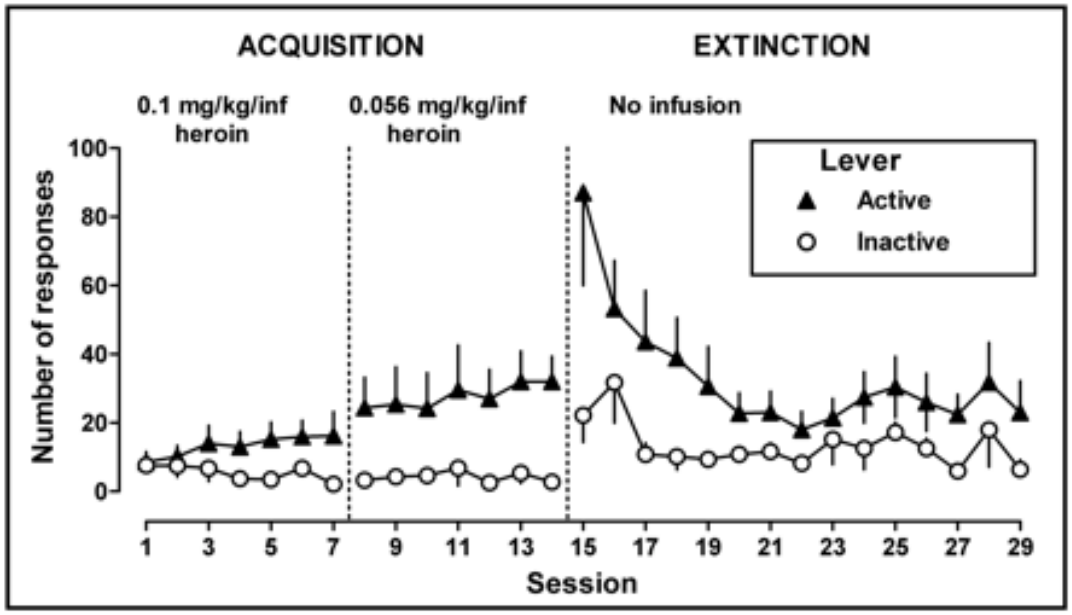


Figure 15. Acquisition and Extinction of heroin self-administration under the FR 1 schedule (Post-reinforcement Time-Out = 2.3 sec; session duration 3 hours). Ordinates: number of responses; Abscissae: sessions. Data are means ± SEMs; N=5 subjects.

Key Research Accomplishments

* One manuscript has been submitted for publication at *Molecular Psychiatry* documenting the efficacy of targeting TLR4 to decreasing cocaine reward/reinforcement. It was reviewed very favorably and the invited revision (now under review) expected to be accepted near term.

* The work has been presented as part of multiple invited research seminars by the PI at conferences and universities

Reportable outcomes

* The work is part of Alexis Northcutt's PhD dissertation research project, successfully defended and her PhD awarded.

* One manuscript has been submitted for publication at *Molecular Psychiatry*. It was reviewed very favorably and the invited revision (now under review) expected to be accepted near term.

*The work has been presented as part of multiple invited research seminars by the PI at conferences and universities

PUBLICATIONS

Wang, X., Cochran, T.A., Hutchinson, M.R., Yin, H., and Watkins, L.R., Microglia and drug abuse, Microglia in Health and Disease, Springer, (2014) in press.

Watkins, L.R., Wang, X., Mustafa, S., & Hutchinson, M.R., *In vivo veritas*: (+)-Naltrexone's actions define translational importance, Trends in Pharmacological Sciences, 35 (2014) 432-433.

Northcutt, A.L., Hutchinson, M.R., Wang, X., Barrata, M.V., Hiranita, T., Cochran, T.A., Pomrenze, M.B., Galer, E.L., Kopajtic, T.A., Li, C.M., Amat, J., Larson, G., Cooper, D.C., Huang, Y., O'Neill, C.E., Yin, H., Zahniser, N.R., Katz, J.L., Rice, K.C., Maier, S.F., Bachtell, R.K., Watkins, L.R., DAT isn't all that: cocaine reward requires toll like receptor 4 signaling, Molecular Psychiatry, (2014) invited revision in review.

Bachtell, R.K., Hutchinson, M.R., Wang, X., Rice, K., Maier, S.F. and Watkins, L.R., Targeting the toll of drug abuse: the translational potential of toll-like receptor 4, CNS & Neurological Disorders: Drug Targets, (2014) in review.

CONFERENCE ABSTRACTS

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Northcutt, A.L., Galer, E.L., Cochran, T.A., Hutchinson, M.R., O'Neill, C.E., Miles, N.E., Haas, M.E., Rozeske, R.R., Amat, J., Maier, S.F., Bachtell, R.K., Rice, K.C., Watkins, L.R., Toll like receptor 4 (TLR4) antagonism suppresses cocaine reward, NIDA Miniconvention, 2012.

Northcutt, A.L., Galer, E.L., Cochran, T.A., Hutchinson, M.R., O'Neill, C.E., Ramos, K.M., Miles, N. Haas, M.E., Rozeske, R.R., Amat, J., Maier, S.F., Bachtell, R.K., Rice, K.C. & Watkins, L.R., Blockade of cocaine-induced proinflammation via toll like receptor 4 (TLR4): antagonism suppresses cocaine-induced conditioned place preference, Society for Neuroscience, 2012.

Fabisiak, T.J., Northcutt, A.L., Cochran, T.A., Weber, M.D., Hutchinson, M.R., Maier, S.F., Rice, K.C., Bachtell, R.K., Watkins, L.R., Exploring whether cocaine-induced priming of innate immune signaling is TLR4 mediated and contributes to sensitized dopamine responsiveness. Proc. Society of Neuroscience, 2014

Conclusions

We continue to make excellent progress and the data to date are clearly supportive that TLR4 is an important drug abuse target. Toll like receptor 4 (TLR4) continues to present as a powerful modulator of drug abuse as blocking TLR4 with (+)naltrexone or (+)-naloxone suppresses multiple indices of drug reward and drug reinforcement for both opioids and cocaine. It is exciting that we are documenting the sites of action of TLR4 on drug abuse to the NAc shell and VTA, key structures in the rewarding and reinforcing effects of cocaine and opioids, the abused drugs under study. As (+)-naltrexone is in preclinical development aiming at human clinical trials, this is especially exciting to have a blood brain barrier permeable, highly selective TLR4 antagonist that would be orally available, stable at room temperature, and appropriate for use from front lines through long-term use. As Xalud Therapeutics received $2.7 million from the Army to move (+)-naltrexone toward FDA Investigational New Drug status, it will be ready for entry into human clinical trials near term. Given our ongoing results, this would be a spectacular step forward for treating warfighters and veterans alike for drug abuse indications.